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USF is a family of transcription factors whose function in growth control can antagonize the transforming activity of the Myc oncoproteins. We are investigating the role of USF in breast cancer. Normal USF DNA-binding activities were observed in a variety of breast cancer cell lines. However, cotransfection experiments revealed a complete lack of USF transcriptional activity in at least three different breast cancer cell lines. This result suggests that, like the overexpression of Myc, the functional inactivation of USF may play an important role in breast cancer by favoring rapid proliferation rates.

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Grant DMAD17-96-1-6221, Role of USF in breast cancer
Michele Sawadogo, Ph.D., Principal Investigator
Progress report: September 1, 1996-August 31, 1997.

INTRODUCTION

We are studying the role of the USF proteins in breast cancer. Reasons to initiate this investigation included the known importance of the Myc oncoproteins in cancer progression in general (1, 2), and as a strong indicator of an increased risk of relapse in the case of breast cancer (3, 4). Both Myc and USF are transcription factors that belong to the basic-helix-loop-helix-leucine zipper family and interact with specific sites on the DNA containing a 5' CACGTG 3' core sequence (5-8). It is well established that the biological function of Myc, which is indispensable during embryonic developement, is to promote cellular proliferation. This effect of Myc on proliferation can lead to uncontrolled growth in transformed cells that overexpress Myc due to gene amplification, translocation, or increased message stability. In contrast, in vitro studies in our laboratory indicated an antiproliferative role of the USF proteins. In particular, we showed that USF was capable of specifically inhibiting the cellular transformation of primary embryo fibroblasts mediated by overexpression of c-Myc and activated Ras (9). Also, constitutive expression of certain forms of USF could inhibit the proliferation of several transformed cell lines. These results suggested that one of the normal functions of USF may be to protect the cells against the oncogenic potential of the Myc oncoproteins (9).

We therefore proposed to:

Specific aim #1: Investigate the expression of the various USF isoforms in breast cancer cells from different origins.

Specific aim #2: Determine whether overexpression of USF could inhibit the proliferation of breast cancer cells, which could open a novel opportunity for gene therapy.

Accomplishment of these specific aims requires the following tasks:

1) Obtain different breast cancer cell lines, expand the original aliquot, and freeze for long term conservation.

2) Characterize the forms of USF present in different breast cancer cells, using nuclear extracts and electrophoretic mobility shift assays.

3) Optimize transfection methods to be used with different breast cancer cell lines.

4) Establish and characterize breast cancer cell lines overexpressing USF.

As detailed below, we have made significant progress towards on all of these tasks. In addition, we discovered that USF is commonly inactivated in breast cancer cells. This

result has opened entirely new areas of investigations, which in the future may lead to the discovery of a new tumor suppressor gene.

BODY

Tasks accomplished:

Task 1: We have now cultured, frozen aliquots, and analyzed four different breast cancer cell lines (MCF-7, BT-20, Hs578T, and MDA-MB-468), and we are getting ready to begin complementary work on two normal breast cells lines, Hs578Bst and HBL-100.
Task 2: We have prepared cytoplasmic and nuclear extracts from the above mentioned breast cancer cell lines, and analyzed the levels of endogenous USF in these extracts.
Task 3: We have used different transfection methods for the four breast cancer lines mentioned above and have in each case defined the best conditions for optimum transfection efficiency.
Task 4: Using these optimized conditions, we have carried out both transient and stable transfection experiments with USF expression vectors in the different cell lines.

Expression of the different USF isoforms in various breast cancer cell lines (specific aim 1):

Our analysis did not reveal any significant difference in the abundance or composition of the various USF1 and USF2-containing homo- and heterodimers in different breast cancer cell lines as compared to other cell lines of human origin such as HeLa and Saos-2 (see Fig. 1). This result indicates that if the USF plays an important role during breast cancer progression, this role does not involve a change in the DNA-binding activity of USF.

Activity of overexpressed USF in breast cancer cell lines (specific aim 2):

Cotransfection experiments in different breast cancer cell lines have yielded novel and important findings.
a) USF is entirely inactive in many breast cancer cell lines: In HeLa cells where they were initially discovered, the USF proteins are transcriptionally active. Consequently, overexpression of either USF1 or USF2 in these cells activates very strongly the transcription of cotransfected USF-dependent reporter genes (12). USF overexpression in HeLa cells also causes a pronounced inhibition of growth (9). In contrast, we have now found that there are cells lines in which USF is entirely inactive in both transcriptional activation and growth control. We initially discovered this phenomenon in the

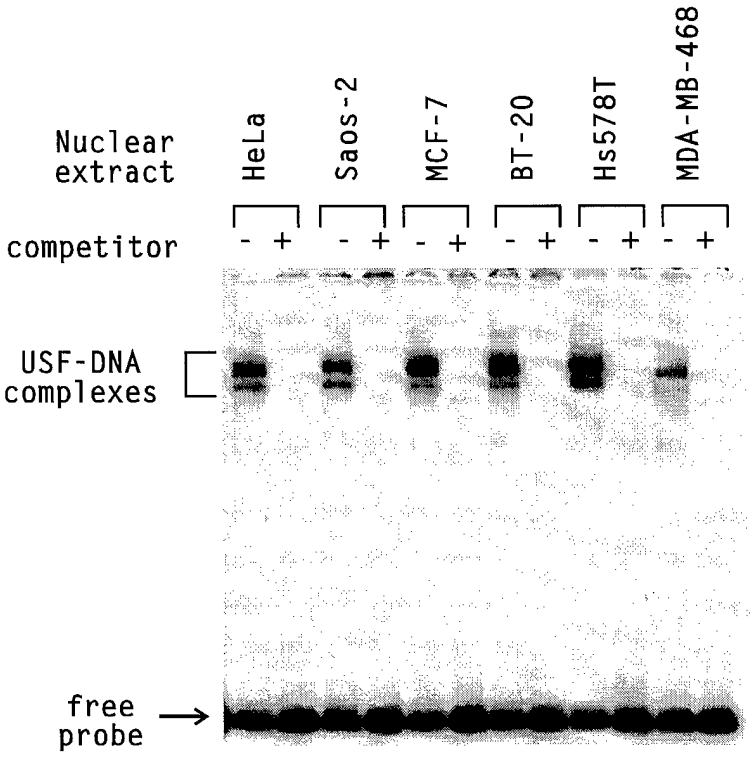

Figure 1: Analysis of USF DNA-binding activity by electrophoretic mobility shift assay in nuclear extracts from different human cell lines. Mini nuclear extracts (10) were prepared from MCF-7, BT-20, Hs578T, and MDA-MB-468 breast cancer cells, and, for comparison, HeLa and Saos2, where USF activity was previously characterized. Extracts were made in each case from 10^6 cells and identical volumes of extracts were used in each reaction. DNA-binding reactions were assembled under optimum conditions for USF binding using as a probe a 150-bp radiolabeled DNA fragment containing a USF-specific binding site. This probe and the gel electrophoresis conditions were chosen to optimize resolution of the different USF dimers (11). Competitor DNA under the form of a oligonucleotide containing the USF consensus binding site was included in the reactions as indicated.

osteosarcoma cell line Saos-2, where we characterized it in greater detail (14). For example, various controls demonstrated that there was no difference in the subcellular localization or DNA-binding activity of USF in HeLa versus Saos-2 cells. Furthermore, a USF2-VP16 fusion construct activated transcription in Saos-2 cells as efficiently as it did in HeLa cells. Normal activity of the transcription activation domains present in the USF1 and USF2 proteins could also be demonstrated in Saos-2 cells when they were fused to the DNA-binding domain of Gal4 (14). From these experiments, we concluded that the transcriptional activity of USF, and consequently its antiproliferative activity, is normally tightly controlled either by posttranslational modification or by interaction with

specific corepressors or coactivators that affect both USF1 and USF2. In contrast to USF itself, these USF-specific effectors are not ubiquitous. The activity of USF is therefore context-dependent, and evidence for USF DNA-binding activity in particular cells is insufficient to indicate USF function in transcriptional activation and growth control (14).

Our results so far indicate that the same lack of USF activity demonstrated in Saos-2 cells is also present in at least three different breast cancer cell lines (MCF-7, Hs578T and MDA-MB-468). In all three cases, cotransfection of USF1 or USF2 had no effect on the transcription of the pU3MLLuc reporter construct (12), which contains three USF binding sites upstream of the adenovirus major late promoter driving luciferase (see Table 1). In contrast, transcriptional activation by the viral IE62 protein, which requires its interaction with either USF1 or USF2 specifically bound to the promoter DNA (13), could be readily demonstrated. Other controls for the efficiency of our transfections in different breast cancer cell lines included the overexpression of the transfected USF proteins (see Fig. 2) and the normal activity of different control reporter genes driven by the SV40 (pSV2-Luc) or RSV (pRSV-Luc) promoters (Table 1).

Transfected plasmids:	HeLa	Saos-2	MCF-7	Hs578T	MDA-MB-468
pU3MLLuc + pSG5	5	35	59	6	9
pU3MLLuc + psvUSF1	1983	25	122	16	9
pU3MLLuc + psvUSF2	1926	36	104	7	7
pU3MLLuc + pcmvIE62	9410	1824	5160	4273	1475
pSV2-Luc	88	91	757	n.d.	n.d.
pRSV-Luc	n.d.	n.d.	n.d.	2523	n.d.

Table 1: Transient cotransfection assays reveal a complete lack of USF transcriptional activity in Saos-2 and in several breast cancer cell lines, while transcriptional activation by both USF1 and USF2 is evident in HeLa cells. The results of the luciferase assays were expressed in arbitrary units and averaged from different experiments. n.d.: not determined.

These results indicate that USF is commonly inactivated in breast cancer cells. Next, we will carry out experiments to determine whether USF is normally active in cells derived from normal breast tissue, which can be done by using for instance Hs578Bst cells or HBL-100 cells at an early passage. If we find USF active in normal breast cells but not in breast cancer cells, this result will indicate that USF inactivation is an

important event in breast cancer progression, probably by triggering uncontrolled growth. If we find that, for some reason, USF is never active in breast cells, this result will suggest that the absence in this tissue of one the most potent antagonists of Myc-mediated cellular transformation may contribute to the frequency at which cancer develops in this particular background.

b) Overexpression of USF in transiently transfected Hs578T breast cancer cells allows vizualization of a previously unknown USF-interacting protein: One control that we routinely use to demonstrate the efficiency of our transfections is to monitor the USF overexpression in the transfected cells by electrophoretic mobility shift assay in the same extracts used to determine luciferase activities. Interestingly, these experiments revealed, in the case of several breast cancer cell lines but most obviously in Hs578T cells, the existence a previously unknown factor that can interact with DNA-bound USF (Fig. 2). The nature of this candidate coeffector of USF, and whether it is involved in the transcriptional inactivity of the USF proteins in breast cancer cells, remain to be determined.

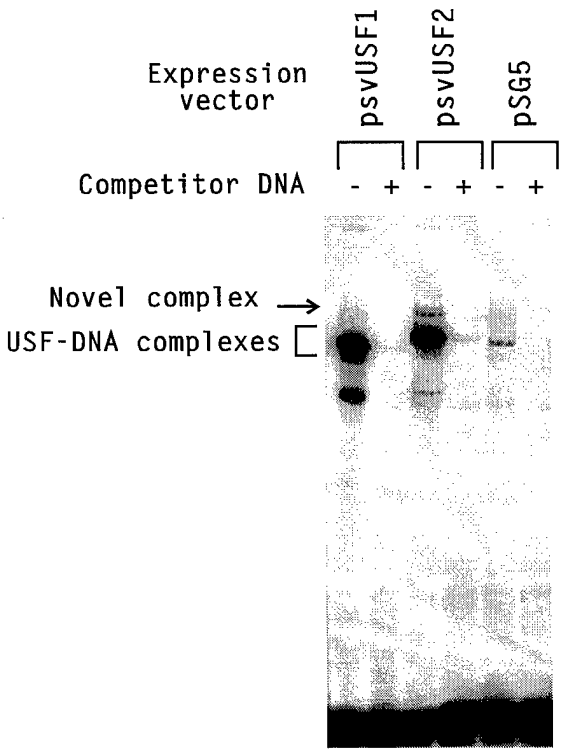

Figure 2: Overexpression of USF2 in Hs578T cells reveals the existence of an unknown USF-interacting protein that is particularly abundant in these cells. Hs578T cells were transiently transfected with vectors driving overexpression of USF1 (lanes 1 and 2) or USF2 (lanes 3 and 4) or with the corresponding empty pSG5 vector (lanes 5 and 6). Whole cell lyzates were used in electrophoretic mobility shift assay with a radiolabeled oligonucleotide probe containing a USF specific binding site. Competitor DNA was added in different reactions as indicated. The bracket indicates the normal migration of the USF1-DNA and USF2-DNA complexes while the arrow indicates the migration of a novel complex that contain USF2 and another protein, as determined by antibody supershift assays (not shown).

Screens for USF-interacting proteins:

We are currently using a yeast two-hybrid system (15) to isolate proteins that specifically interact with either USF1, USF2, or both. These screens have already yielded several interesting candidate genes. Our plan will be to determine in the future whether one of these USF-interacting proteins is involved in the specific inactivation of USF in breast cancer cells.

Publications:

A first manuscript acknowledging support from the Department of the Army has been recently submitted for publication (see reference 14).

CONCLUSIONS

We have analyzed the abundance and activity of the USF proteins in breast cancer cells. Both USF1 and USF2 polypeptides that are active in DNA-binding are present at normal levels in these cells as compared to other human cell lines such as HeLa. However, in contrast to the results previously obtained in HeLa cells, USF was found to be transcriptionally inactive in many breast cancer cell lines. This lack of USF activity in breast cancer cells is probably an important factor contributing to their tumorigenicity. Further studies will be necessary to determine the genetic alteration(s) that are responsible for the inactivity of USF in different cancer cells and whether these involve a novel tumor suppressor gene.

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